INHIBITION BY PLANT GROWTH RETARDANTS OF CHOLESTEROL BIOSYNTHESIS IN SLICES OF RAT LIVER AND HEPATOMA

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Abstract

The plant growth retardant Phosfon inhibits cholesterol formation by cell-free preparations of normal liver and the Morris hepatomas 5123C, 7794A, and 9618A. In addition, both Phosfon and Amo 1618 are active in inhibiting cholesterol synthesis from acetate and mevalonate in slices of host liver and the hepatomas. Fatty acid synthesis from acetate was also reduced in the presence of the retardants.

I. Introduction

Inhibition of mammalian sterol biosynthesis is usually accomplished in two distinct ways. First, there are a group of chemicals which have been shown to affect at least one, and usually more than one, particular enzyme step in the biosynthetic sequence (Holmes and Bentz 1960; Holmes and Di Tullio 1962). The second type of inhibition is produced in an, as yet, undefined manner by certain dietary factors such as high cholesterol diets, starvation, etc. (Sabine, Abraham, and Chaikoff 1967; Sabine, Abraham, and Morris 1968; Zuckerman *et al.* 1970). Dietary factors are particularly effective in inhibiting cholesterol biosynthesis in normal livers, but appear to lack any control function in the regulation of cholesterol biosynthesis in either primary or transplanted hepatomas. The reason for the difference in sensitivity between normal and cancerous hepatic tissue is not clear.

Recently, several publications (Paleg 1970*a*, 1970*b*) have demonstrated that a group of structurally unrelated chemicals, collectively called "plant growth retardants", have the capacity to inhibit cholesterol synthesis by cell-free preparations derived from normal rat livers. In common with the SK&F series of sterol synthesis inhibitors, several of the retardants possess a quaternary nitrogen; other members of the group, however, have phosphonium ions or other types of active configurations (Cathey 1964).

Since the plant growth retardants produce agriculturally desirable crop responses, and since, also, they have been shown to inhibit mammalian cholesterol biosynthesis, it was decided to investigate further their effects on mammalian systems. The results of this work report the extension of retardant (Amo 1618 and Phosfon) effectiveness to whole-cell preparations in the form of liver slices, and the similarity in effect of the retardants on normal liver and three "minimal-deviation" hepatomas.

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II. Materials and Methods

(a) *Animals and Diets*

Adult male rats of the Buffalo strain were used, each carrying intramuscularly one of the Morris hepatomas—5123C, 7794A, or 9618A. Our original supply of these hepatomas and our original breeding stock of Buffalo-strain rats were generously donated by Dr. H. P. Morris, formerly of the National Cancer Institute, Bethesda, Md., U.S.A. The tumor-bearing animals used in these studies either had been air-freighted from the United States some months prior to an experiment or were subsequent transplant generations produced in Adelaide. All animals received a standard laboratory diet (Charlicks' M164 mouse cubes) and water *ad libitum*.

(b) *Plant Growth Retardants*

Phosfon (tributyl-2,4-dichlorobenzylphosphonium chloride) was a gift from the Mobil Chemical Company, Ashland, Va., U.S.A., and Amo 1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride) was purchased from Calbiochem. These were dissolved in saline before addition to the incubations.

(c) *Tissue Incubations*

The standard procedures used for the preparation and incubation of tissue homogenates (Paleg 1970*a*, 1970*b*) and slices (Sabine, Abraham, and Chaikoff 1967) and for the collection, isolation, and counting of ^{14}C-labelled cholesterol (as the digitonin precipitate) and long-chain fatty acids (Sabine, Abraham, and Chaikoff 1967; Paleg 1970*a*, 1970*b*) have been described elsewhere. Sodium [1-^{14}C]acetate and DL-[2-^{14}C]mevalonic acid lactone were obtained from the Radiochemical Centre, Amersham, England and dissolved in saline for use in the incubations.

III. Results

(a) *Tissue Homogenates*

The inhibiting effect of Phosfon on cholesterol synthesis from mevalonate is shown in Table 1. The results demonstrate that cholesterol synthesis by homogenates

Table 1

INHIBITION BY PHOSFON OF CHOLESTEROL SYNTHESIS FROM [2-^{14}C]MEVALONATE BY HOMOGENATES OF RAT LIVER AND HEPATOMA

Homogenates (representing approximately 2 g wet weight of tissue) were incubated with DL-[2-^{14}C]mevalonate (4 μmoles), in the presence of varying amounts of Phosfon. Three experiments are shown, with tissue from one animal in each experiment. Values in parentheses show percentage inhibition

Tissue	Phosfon (μg/ml)	Percentage Recovery of ^{14}C in Cholesterol from		
		5123C*	7794A*	9618A*
Host liver	0	2·32	22·75	19·46
	10	1·71 (26)	21·49 (6)	19·69
	1000	0·68 (71)	1·91 (92)	2·80 (86)
Hepatoma	0	0·69	2·75	8·79
	10	0·26 (62)	2·18 (21)	9·78
	1000	0·03 (96)	0·23 (92)	0·22 (97)

* Hepatoma numbers.

of three different hepatomas can be inhibited by this retardant, and that the degree of inhibition was approximately the same for both host liver and hepatoma.

(*b*) *Tissue Slices*

In addition to their effects on tissue homogenates, Phosfon (Table 2) and Amo 1618 (Table 3) inhibit the conversion of [1-^{14}C]acetate and [2-^{14}C]mevalonate to cholesterol and long-chain fatty acids by tissue slices. Cholesterol synthesis in both liver and hepatoma was reduced by the addition of retardant and, furthermore, the retardants (particularly Phosfon) reduced the incorporation of acetate into long-chain fatty acids as well as into cholesterol. This is the first indication of an effect of these compounds in a mammalian system on a synthetic pathway other than that for cholesterol formation.

TABLE 2

EFFECTS OF PHOSFON ON THE CONVERSION OF [1-^{14}C]ACETATE AND [2-^{14}C]MEVALONATE TO CHOLESTEROL AND LONG-CHAIN FATTY ACIDS BY SLICES OF RAT LIVER AND HEPATOMA

200 mg of tissue was incubated under an atmosphere of 95% O_2 : 5% CO_2 for 2 hr at 37°C in 2·0 ml buffer, plus labelled substrate (4 μmoles), with and without Phosfon (500 μg/ml). Three experiments are shown, each involving tissues from one animal. Values given are means of duplicate determinations, with percentage inhibition shown in parentheses

Tissue	Phosfon	Percentage Recovery of ^{14}C from [1-^{14}C]Acetate in		Percentage Recovery of ^{14}C from [2-^{14}C]Mevalonate in	
		Cholesterol	Long-chain Fatty Acids*	Cholesterol	Long-chain Fatty Acids*
Host liver	—	0·52	0·81	1·78	1·41
	+	0·10 (81)	0·35 (57)	0·84 (53)	1·24 (12)
Hepatoma (7794A)	—	0·10	1·54	1·70	1·36
	+	0·01 (90)	0·46 (70)	0·52 (69)	0·89 (35)
Host liver	—	0·14	3·15	5·16	1·51
	+	0·01 (93)	0·19 (94)	2·64 (49)	1·86
Hepatoma (9618A)	—	0·24	3·41	2·58	0·89
	+	0·05 (79)	0·67 (80)	1·21 (53)	0·86 (3)
Host liver†	—	0·01	0·24	0·41	0·37
	+	0·01	0·24	0·20 (51)	0·34 (8)
Hepatoma (9618A)†	—	0·65	1·87	1·60	0·83
	+	0·11 (83)	0·87 (53)	0·83 (48)	0·85

* Possibly various terpenoids rather than fatty acids.

† In this experiment the incubation atmosphere was air.

A proportion of radioactivity from mevalonate appeared in the long-chain fatty acid fraction. As yet, we have no explanation for this, although Christophe and Popjack (1961) and Carroll (1964) have suggested that radioactivity in the "fatty-acid fraction" after incubation with ^{14}C-mevalonate may be in terpenoid acids, byproducts of the sterol biosynthesis pathway. No attempt has been made to identify the labelled compound(s) present, although we have found that mevalonate itself does

not appear in this fraction. The data indicate, however, that incorporation of radioactivity from [^{14}C]mevalonate into this fraction was not sensitive to the presence of the retardants.

TABLE 3

EFFECTS OF AMO 1618 ON THE CONVERSION OF [1-^{14}C]ACETATE TO CHOLESTEROL AND LONG-CHAIN FATTY ACIDS BY SLICES OF RAT LIVER AND HEPATOMA

Three experiments are shown and each involved tissues from one animal. Values given are means of duplicate determinations, with percentage inhibition shown in parentheses. Experimental conditions are as in Table 2, except that the concentration of retardant was either 100 or 1000 μg/ml

Tissue	Amo 1618 (μg/ml)	Percentage Recovery of ^{14}C in	
		Cholesterol	Long-chain Fatty Acids
Host liver	—	0·30	4·38
	100	0·24 (20)	4·46
Hepatoma	—	0·24	0·75
(5123C)	100	0·10 (58)	0·62 (17)
Host liver	—	0·21	1·01
	100	0·09 (57)	0·61 (40)
	1000	0·01 (95)	0·57 (44)
Hepatoma	—	0·05	0·89
(7794A)	100	0·05	0·88 (1)
	1000	0·02 (60)	0·73 (18)
Host liver	—	0·26	1·71
	100	0·03 (88)	1·18 (31)
	1000	0·01 (96)	0·46 (73)
Hepatoma	—	0·53	8·60
(9618A)	100	0·53	8·94
	1000	0·16 (70)	6·44 (25)

IV. DISCUSSION

An important aspect of the potential effect of plant growth retardants on intact mammalian systems is their ability to affect whole cells. In this regard, the present results strongly suggest that plant growth retardants are potent sterol biosynthesis inhibitors when acting on intact cells, in the form of liver slices, as well as in cell-free liver preparations.

In common with the well-known sterol biosynthesis inhibitors, e.g. the SK&F compounds, the plant growth retardants inhibit at least one step between acetate and mevalonate and at least one step between mevalonate and cholesterol (Holmes and Di Tullio 1962; Paleg 1970*a*, 1970*b*). The precise step which is inhibited by each retardant, in each section of the biosynthetic sequence, has not been determined, although definitive evidence has been obtained, in as yet unpublished experiments, demonstrating that Amo 1618 inhibits the cyclization of squalene-2,3-epoxide. It seems highly probable that Amo 1618 has the same effect in the cells of liver slices.

The nature of the lesion in the control of lipid metabolism in hepatomas is, as yet, unknown, although many recent papers (Sabine, Abraham, and Chaikoff 1967; Sabine, Abraham, and Morris 1968; Sabine 1969; Zuckerman *et al.* 1970) have clearly illustrated that cholesterol synthesis in both primary and transplanted hepatomas is not affected by factors that regulate it in normal liver. The present work indicates that the two growth retardants, Phosfon and Amo 1618, not only affect cholesterol synthesis in three hepatomas as easily as they inhibit it in normal liver, but they also inhibit it to about the same extent in all four tissues. This suggests that the enzymic steps which are affected are the same in the normal rat liver and the hepatoma. Obviously, this brief investigation is not sufficient to negate the possibility that the biosynthetic sequence in liver and hepatoma is different. However, it does lend support to the suggestion that differential control of cholesterol synthesis in the liver and the cancerous tissue is vested in some other type of mechanism (Sabine 1969), although much further work is needed with a range of sterol biosynthesis inhibitors to adequately determine differences in enzyme pathways or sensitivities between the normal and the cancerous tissue or both. Although it does not seem to have been used before, the technique of assessing with inhibitors the sterol biosynthetic pathway of hepatomas appears to offer certain advantages.

These findings emphasize the need for further research into the *in vivo* effect of plant growth retardants on mammalian systems, and suggest that these compounds may be useful in helping to elucidate the mechanisms controlling cholesterol synthesis.

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